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Determinants of the essential one-carbon metabolism metabolites homocysteine, S-adenosylmethionine, S-adenosylhomocysteine, and folate in cerebrospinal fluid.

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Abstract

Background: Disturbances in the levels of one-carbon (1C) metabolism metabolites have been associated with a wide variety of neuropsychiatric diseases. CSF levels of Hcy and the other 1C metabolites, nor their interrelatedness and putative determinants, have been studied extensively in a healthy population.

Methods: Plasma and CSF samples from 100 individuals free from neuropsychiatric diseases were analyzed (55 male, 45 female; age 50 ± 17 years). In blood, we measured plasma Hcy, serum folate and serum vitamin B12. In CSF, we measured total Hcy, SAM, SAH and 5-methylTHF. Highly selective analytical methods like liquid chromatography combined with either mass spectrometry or fluorescence detection were used.

Results: CSF Hcy was inversely correlated with CSF 5-methylTHF and positively with plasma Hcy, independent of serum folate status. CSF SAH correlated with age, lower CSF 5-methylTHF, and higher CSF Hcy. CSF 5-methylTHF showed independent negative correlations with age, and positive correlations with serum folate. CSF SAM did not correlate with any of the 1C metabolites.

Conclusions: Ageing is characterized by a reduction in CSF 5-methylTHF levels and increased CSF levels of the potentially neurotoxic transmethylation inhibitor SAH. CSF 5-methylTHF, which is itself determined in part by systemic folate status, is a powerful independent determinant of CSF levels of Hcy and SAH.

Introduction

One-carbon (1C) metabolism encompasses a series of biochemical reactions that involves the transfer of 1C moieties (Figure 1). One of its main purposes is to provide methyl groups for virtually all transmethylation reactions. 1C metabolism has been the focus of extensive research, particularly since increased plasma levels of one of its intermediates, homocysteine (Hcy), were linked to numerous diseases, such as cardiovascular disease (1) and neurological disease. (3,4).

Studies with small sample sizes have also shown correlations of these neurological diseases with Hcy in cerebrospinal fluid (CSF).(5;6) To date, however, several uncertainties surround the association between increased plasma levels of Hcy and neuropsychiatric disease. Firstly, it is unclear whether plasma or CSF concentrations of these constituents are primarily of interest. In addition, it remains unclear whether Hcy itself or one of the other constituents of 1C metabolism exerts detrimental effects on the neural system. Potential candidates in this context include S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and B-vitamin status.

SAM is the methyl donor in many essential methylation reactions such as DNA methylation, synthesis and inactivation of neurotransmitters and myelin synthesis. SAH has opposite effects, in that it acts as a powerful inhibitor of most transmethylation reactions.(7) The SAM/SAH ratio has been used as a reflection of methylation potential in, amongst others, the central nervous system (CNS).(8) The potential relevance of SAM is supported by observations that low SAM levels in CSF are associated with depression and dementia.(9) The effect of SAH on

neuropsychiatric disturbances is less well studied. However, the CSF levels of SAH are associated with CSF levels of hyperphosphorylated tau, a marker for Alzheimer pathology (10).

One-carbon metabolism is critically dependent on the B-vitamin status (B2, B6, B12 and, predominantly, folate).(11) Deficiencies of B-vitamins are commonly seen in the elderly population.(12;13) Since the remethylation pathway via betaine is not expressed in the brain, the only option for remethylation of Hcy, and thus regeneration of methionine, is via 5-methyltetrahydrofolate (5-methylTHF; Figure 1). Therefore, low 5-methylTHF levels could not only increase Hcy levels but could also potentially diminish the methylation capacity of the brain.(14) Additionally, folates are involved in DNA synthesis, and low folate levels could potentially impair DNA stability and structure, for example by the misincorporation of uracil in DNA.(15) The literature on folate status as an independent risk factor for neuropsychiatric diseases indicates that low levels of 5-methylTHF in CSF are found in patients suffering from a wide spectrum of neuropsychiatric diseases such as Alzheimer, depression, and autism.(16-19) Additionally, the absence of 5-methylTHF in CSF in rare inborn errors of metabolism causes progressive neurological decline.(20)

In short, concentrations in the CNS of Hcy, but also of other 1C metabolites, in particular SAM, SAH and 5-methylTHF, may be of relevance for the development of neuropsychiatric diseases. A better understanding of the determinants of these intermediates is important. This paper focuses on identification of determinants of Hcy, SAM, SAH and 5-methylTHF in CSF of individuals unaffected by neuropsychiatric disease. Additionally, the reference values in this paper will offer an indication for the sample size estimation of future studies.

Materials and methods

Subjects

Ninety eight subjects, who were subjected to a lumbar puncture (for different indications such as exclusion of CNS inflammation, exclusion of aneurysmal subarachnoid hemorrhage or exclusion of meningitis, all negative) at the Department of Neurology (University of Bonn), were enrolled in this study. Except for the symptoms they presented with – majorly acute, but not chronic cepahlgia – none of the enrolled subjects showed evidence or history of cognitive impairment or any other symptoms of possible neurological or psychiatric disease, which were exclusion criteria. Additional exclusion criteria for this study were: chronic or unstable medical illness (e.g. symptomatic cardiac disease, renal or hepatic dysfunction, insulin-dependent diabetes mellitus, untreated thyroidal dysfunction), excessive alcohol intake, vitamin supplementation, disturbance of the blood brain-barrier (defined as CSF whole protein content >500mg/dL), or inflammation of the central nervous system (i.e. more than 5 leucocytes/mm^3 or intrathecal immunglobuline production) or an abnormal result in the Mini Mental State Examination (MMSE), which was done for all subjects (21). The study was approved by the local ethics committee, and written informed consent was obtained from all study participants.

Sample collection

Diagnostic lumbar punctures were performed at the Department of Neurology, University of Bonn. A standardized technique with a 20G “atraumatical” spinal needle

and a sitting or lying position for the patient was applied. Approximately 10 to 14 ml of CSF was removed for analysis. Depending on the indication of the lumbar puncture, the first fractions were used for diagnostic purposes, the third or fourth for the current study. CSF samples were frozen immediately on dry ice.

Fasting venous blood was collected in lithium heparine vacutainers, and immediately placed on ice. Plasma was isolated by centrifugation for 10 min at 2000 g at 4ºC. Light protected sera were used for the folate and vitamin B12 measurements which were centrifuged for 10 min at 3300 g at room temperature. All samples were stored at -80ºC until analysis.

Methods

Plasma total Hcy concentrations were determined by means of fully automated particle-enhanced immunonephelometry with a BN II System (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). The intraassay and interassay coefficient of variation (CV) of the homocysteine assay were 3.4% and 5.6 %, respectively.

Serum vitamin B12 and folate concentrations were determined by means of a competitive chemiluminescent immunoassay with an Access™ Immunoassay System (Beckman Coulter, Krefeld, Germany). The intraassay and interassay CVs of the vitamin B12 assay were 3.8% and 6.3 %, respectively. The intraassay and interassay CVs of the folate assay were 2.8% and 4.8 %, respectively.

CSF SAM and SAH concentrations were determined by LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). The intraassay and interassay CVs for SAM were 6.8% and 4.2%, respectively. The intraassay and interassay CVs for SAH were 6.9% and 5.5%, respectively.(22)

CSF folate vitamers were determined by LC-MS/MS (API3000, Applied Biosystems). Intraassay and interassay CVs for 5-methyltetrahydrofolate (5-methylTHF) were 1.2% and 2.8%, respectively. Intraassay and interassay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively.(23)

CSF total Hcy concentrations were measured by HPLC using fluorescence detection (Waters, Milford, MA, USA). An adaptation of a previously published method was used.(24) In order to be able to measure the nanomolar homocysteine concentration in 100 µL of CSF accurately, the reaction volume was minimized, and a flushing gradient (30% acetonitrile) was added after each injection to create a more stable HPLC baseline. The intraassay and interassay CVs were 2.2% and 3.6% respectively.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 for Windows. Most of the measured parameters showed positively skewed distributions (plasma Hcy, CSF Hcy, serum folate, serum B12, CSF SAH, CSF SAM/SAH), so the data were log transformed prior to regression analysis. As outlined in the introduction, the main dependent variables of interest in CSF were Hcy, SAM, SAH, SAM/SAH and 5-methylTHF. We performed separate analyses to identify determinants of these CSF constituents.

In order to reduce the risk of finding associations by chance, a priori hypotheses regarding plausible determinants of the dependent variables of interest were formulated using available knowledge of 1C metabolism and epidemiology. For CSF Hcy, we first assessed traditional determinants known to influence plasma Hcy,

namely age and B-vitamin status (in both plasma and CSF). In order to determine whether serum folate status would determine CSF Hcy via its correlation with CSF folate status, serum folate and CSF 5-methylTHF were analyzed in a multiple regression model. In addition, we analyzed the correlation between plasma versus CSF Hcy, and adjusted associations between serum folate status and CSF Hcy for plasma Hcy. All significant determinants of CSF Hcy were finally entered into a single multiple regression model.

For CSF SAM, SAH and their ratio, the following potential determinants were analyzed: age, serum B vitamin status, plasma and CSF Hcy, and CSF 5-methylTHF. Positive correlations were analyzed for independence, again with multiple regression analysis

Finally, for CSF 5-methylTHF, we assessed whether its concentration independently correlated with age and/or serum folate status, and whether the CSF 5-methylTHF/serum folate ratio correlated with age.

Since most of the measured parameters were log transformed, interpretation of the effect size of a given determinant on the investigated 1C metabolite is difficult. In order to display the effect sizes more clearly, we divided the non logarithmic transformed levels of the investigated metabolites into tertiles, repeated the multivariate analyses, and plotted the increase in the dependent variable in tertiles, with the first tertile as reference.

Results

Characteristics of included individuals are listed in Table1. CSF values for Hcy, folate, SAM, and SAH compared well with a previous reported study.(8) Some individuals showed hyperhomocysteinemia in plasma, which coincided with low serum folate levels. Subjects were not excluded for this reason. With regard to the comparison of 1C metabolites levels in CSF to plasma, CSF Hcy levels were a 100 fold lower than the plasma levels. Remarkably, CSF folate levels were substantially higher than plasma levels. SAM and SAH levels in CSF were comparable to plasma levels, previously determined using similar analytical methods.(25) Of note, folate existed in CSF predominantly in the 5-methylTHF form, as was previously described for plasma.(26) With regard to gender, no significant correlations with any of the parameters were found (data not shown).

Determinants of CSF Hcy

For the potential determinants of CSF Hcy listed in the methods section, the univariate correlations were determined. The results are listed in Table 2. CSF Hcy levels were positively correlated with plasma Hcy and CSF SAH, and negatively correlated with serum folate and CSF folate. Age, a traditional determinant of plasma Hcy, just failed to reach significance for CSF Hcy (P=0.075).

Multiple regression analysis was used to determine interdependence of the univariate correlations. As is evident from both multivariate regression model 1 and 2 in Table 2, serum folate is not an independent determinant of CSF Hcy. Rather, its correlation with CSF Hcy is predominantly explained by its effect on CSF 5-methylTHF and plasma Hcy.

As explained in Figure 1, the metabolic fate of Hcy is determined by several pathways, implying that its concentration could be independently correlated to parameters related to these pathways. Regression model 3 indeed identified CSF 5-methylTHF (remethylation), plasma Hcy (import/export across the blood-brain barrier) and CSF SAH (SAH hydrolase activity) as independent determinants of CSF Hcy levels. When age and vitamin B12 were forced into the multiple regression model, they were again not significantly related to CSF Hcy (data not shown). The Figure 2 demonstrates the multiple adjusted impact of plasma Hcy, CSF 5-methylTHF, and CSF SAH on CSF Hcy.

Determinants of CSF SAM, SAH and SAM/SAH

As shown in Table 3, CSF SAM showed no significant correlation with any of the potential determinants studied. CSF SAH was independently positively correlated with age and CSF Hcy, and negatively correlated with CSF 5-methylTHF (Figure 2). The CSF SAM/SAH ratio was independently positively correlated with CSF 5-methylTHF and negatively correlated with age and CSF Hcy. None of the serum parameters (Hcy, folate and B12) showed any significant correlation with either CSF SAM, SAH or the SAM/SAH ratio (data not shown).

Determinants of CSF 5-methylTHF

As shown in Table 4, CSF 5-methylTHF is negatively correlated with age and positively correlated with serum folate concentrations. A multivariate regression model for both age and serum folate was applied, showing that both parameters independently influence CSF 5-methylTHF (Figure 2). Additionally, the ratio CSF 5-

methylTHF/serum folate was negatively correlated with age (R^2=0.076, ß=-0.276, P=0.008).

Discussion

The salient findings of our study in healthy individuals are that, firstly, older age and lower serum folate status are independently associated with lower CSF 5-methylTHF availability. This may be unfavorable, since we also observed that a low CSF folate status adversely affects CSF Hcy and methylation potential, at least insofar as the latter is reflected by concentrations of SAH. Secondly, aging also appears to directly adversely affect methylation potential, independent of CSF folate status. Finally, CSF Hcy appears to be at least partly determined by plasma Hcy, suggesting a degree of blood-brain equilibration. In the highest tertile of CSF Hcy, the contribution of plasma Hcy is less, suggesting that higher CSF Hcy is predominantly related to intracerebral metabolism, rather than to equilibration with plasma Hcy. CSF Hcy is independently correlated to the concentration of the transmethylation inhibitor SAH, which may represent a mechanism of untoward effects of CSF Hcy.

The fate of Hcy in CSF will depend on many different factors (remethylation, export from brain tissue, transport across the blood-brain barrier, equilibrium with SAH, etc). From the perspective of Hcy metabolism (Figure 1), the positive correlation of CSF Hcy with both plasma Hcy and CSF SAH, and the negative correlation with CSF 5-methylTHF seems logical. (8) However, given the strong degree of inter-relatedness of 1C metabolites, analyzing statistical independency of these associations, as we did in the current study, is crucial.

The methylation potential of cells is conceivably reflected by SAM and SAH (and their ratio), since SAM is a universal methyl donor, and SAH is an inhibitor of most transmethylation reactions. However, in line with previously reported data, CSF SAM levels did not correlate with any of the other studied potential determinants.(8) From a teleological perspective, this could mean that keeping CSF SAM levels stable is vitally important, which would be in keeping with its central role not only as a methyl donor, but also as a regulator of enzyme activity in both the remethylation and transsulfuration pathway. The fact that significantly lower amounts of SAM were measured in CSF of patients suffering from depression and dementia, may offer insight into how disturbances in 1C metabolism and this type of diseases are linked.(9) It may be that intracellular concentrations in brain tissue obtained from specific regions will offer more insight into these matters, since CSF SAM does not correlate well with intracellular SAM concentrations in brain.(27;28)
Levels of the methyltransferase inhibitor SAH were adversely associated with higher age and CSF Hcy.(8) Age seems to be a strong determinant of the methylation potential in the brain. The underlying mechanism remains unclear.

In our study, CSF folate levels were approximately 5 times higher than serum folate levels. Another study (17) showed similar results, while a further one (8) observed higher folate serum levels than in our population, and lower CSF levels. However, this last study did not use multivitamin intake as a exclusion criteria. This may account for the higher plasma values. Perhaps folate demand in the brain is higher than in other organs, but exactly how the brain maintains its high folate status is mechanistically unknown. The relation between increased age and diminished serum folate levels has been well documented.(13) This correlation has been attributed to

the decreased ability for folate uptake in the elderly population (12;13) Our study also showed that age was an independent determinant of CSF folate levels, a fact that has not been described before to our knowledge.

Since, CSF folate levels have been shown not to follow serum folate levels outside the normal range (≥ 45 nmol/L)(8), an additional determinant of CSF folate is of importance. Choroid plexus epithelial cells express high levels of folate receptor α suggesting that a role for it in folate trafficking.(29) Perhaps transport of folate over the blood-brain barrier is also a determinant of CSF folate, and might be the rate limiting step. The observation that the CSF/serum folate ratio diminishes with age may suggest that this transport alters with age. Supplementation might increase the often reduced plasma folate levels of the elderly. However, whether this supplementation will lead to desired increase in CSF folate has yet to be determined. Additionally, this observation of hindered transport of folate across the blood-brain barrier could provide insight to the origin of the low CSF folate levels observed in cerebral folate deficiency, a treatable neurological abnormality with genetic, acquired or even unknown etiology. (30) Whether deficient CSF folate reflects an intracellular folate deficiency in the brain is not well understood. Because of the strong observed correlation between CSF folate and CSF Hcy, CSF Hcy could be considered to be a more practical biomarker (compound stability) of cerebral folate deficiency.

Obtaining CSF samples from healthy volunteers is difficult. Even though individuals taking medication or suffering from diseases known to influence the 1C metabolism were omitted, a certain degree of bias cannot be excluded.

Having identified the determinants of 1C metabolites in CSF, it will be interesting to investigate whether the same determinants play a role in various neurodegenerative

diseases, and whether various intervention strategies can alter CSF concentrations of the relevant 1C metabolites, offering pathways to prevention and/or treatment.

In conclusion, CSF 5-methylTHF levels decrease while CSF SAH levels increase with age, which may contribute to CNS ageing and neurodegeneration. Higher serum folate concentrations were associated with higher CSF concentrations of 5-methylTHF and with lower levels of the neurotoxic agents Hcy and SAH arguing that folate deficiency may accelerate ageing and neurodegeneration.

Table 1: Study population characteristics (data are presented as mean (SD) or, in case of skewed distributions, as median (range)).

Age, years	50 ± 17
Gender, male/female, n	55/45
Plasma homocysteine, μM	11 (4-34)
Serum total folate, nM	11 (2-39)
Serum vitamin B12, pg/mL	325 ± 126
CSF homocysteine, nM	61 (13-303)
CSF 5-methylTHF, nM	42 ± 14
CSF non-methylTHF, nM	0 (0-12)
% non-methylTHF	0 (0-31)
CSF SAM, nM	205 ± 41
CSF SAH, nM	22 (11-49)
CSF SAM/SAH	9 (3-20)

Table 2: Univariate and multivariate regression models of determinants of CSF Hcy.

Regression type	Dependent	Independent	β	P
Univariate	CSF Hcy[c]	Age	0.181	0.075
Univariate	CSF Hcy[c]	CSF 5-methylTHF	-0.495	0.000[b]
Univariate	CSF Hcy[c]	Plasma Hcy[c]	0.331	0.001[b]
Univariate	CSF Hcy[c]	Serum folate[c]	-0.244	0.019[a]
Univariate	CSF Hcy[c]	Serum B12[c]	-0.176	0.094
Univariate	CSF Hcy[c]	CSF SAH[c]	0.380	0.000[b]
Multivariate 1	CSF Hcy[c]	CSF 5-methylTHF	-0.499	0.000[b]
		Serum folate[c]	0.008	0.943
Multivariate 2	CSF Hcy[c]	Plasma Hcy[c]	0.279	0.016[a]
		Serum folate[c]	-0.109	0.339
Multivariate 3	CSF Hcy[c]	CSF 5-methylTHF	-0.338	0.001[b]
		Plasma Hcy[c]	0.204	0.027[a]
		CSF SAH[c]	0.265	0.004[b]

[a] Correlation is significant at the 0.05 level (2-tailed), [b] Correlation is significant at the 0.01 level (2-tailed), [c] Log transformed data.

Table 3: Univariate and multivariate regression models of determinants of CSF SAM, SAH and SAM/SAH.

Regression type	Dependent	Independent	β	P
Univariate	CSF SAM	Age	0.033	0.750
Univariate	CSF SAM	CSF 5-methylTHF	0.093	0.365
Univariate	CSF SAM	CSF Hcy[c]	0.164	0.107
Univariate	CSF SAH[c]	Age	0.462	0.000[b]
Univariate	CSF SAH[c]	CSF 5-methylTHF	-0.321	0.001[b]
Univariate	CSF SAH[c]	CSF Hcy[c]	0.380	0.000[b]
Univariate	CSF SAM/SAH[c]	Age	-0.405	0.000[b]
Univariate	CSF SAM/SAH[c]	CSF 5-methylTHF	0.361	0.000[b]
Univariate	CSF SAM/SAH[c]	CSF Hcy[c]	-0.252	0.012[a]
Multivariate	CSF SAH	Age	0.391	0.000[b]
		CSF 5-methylTHF	-0.097	0.336
		CSF Hcy[c]	0.261	0.010[a]
Multivariate	CSF SAM/SAH	Age	-0.333	0.001[b]
		CSF 5-methylTHF	0.246	0.022[a]
		CSF Hcy[c]	-0.070	0.499

[a] Correlation is significant at the 0.05 level (2-tailed), [b] Correlation is significant at the 0.01 level (2-tailed), [c] Log transformed data. All serum (Hcy, folate and B12) correlations were not significant.

Table 4: Univariate and multivariate regression models of determinants of CSF 5-methylTHF.

Regression type	Dependent	Independent	β	P
Univariate	CSF 5-methylTHF	Age	-0.242	0.016[a]
Univariate	CSF 5-methylTHF	Serum folate[c]	0.504	0.000[b]
Multivariate	CSF 5-methylTHF	Age	-0.256	0.004[b]
		Serum folate[c]	0.511	0.000[b]

[a] Correlation is significant at the 0.05 level (2-tailed), [b] Correlation is significant at the 0.01 level (2-tailed), [c] Log transformed data.

Figure legends

Figure 1: Overview of the 1 carbon metabolic pathway in humans. Homocysteine (Hcy) can follow two pathways. It could be remethylated to methionine, and subsequently converted to the universal methyl donor S-adenosylmethionine (SAM). Disturbed remethylation influences methylation reactions such as the methylation of neurotransmitters or DNA. Alternatively, Hcy can be irreversibly converted to cystathionine and further to cysteine. However, whether this transsulfuration pathway is active in the brain, is still debated. (31) Elevated Hcy levels could also influence SAH levels by the equilibrium reaction. High SAH is an inhibitor of a wide variety of methyltransferases. (32)
(methionine synthase (MS), methylene tetrahydrofolate reductase (MTHFR), cystathionine β-synthase (CBS), methionine adenosyl transferase (MAT), SAH hydrolase (SH)).

Figure 2: Change in CSF Hcy, CSF Folate, and CSF SAH in respective tertiles (multivariate regression model). Effect sizes were adjusted for the variables as indicated in Table 2.

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